THE CONVERSION OF PRESQUALENE PYROPHOSPHATE INTO SQUALENE BY A CELL-FREE PREPARATION OF *PISUM SATIVUM*

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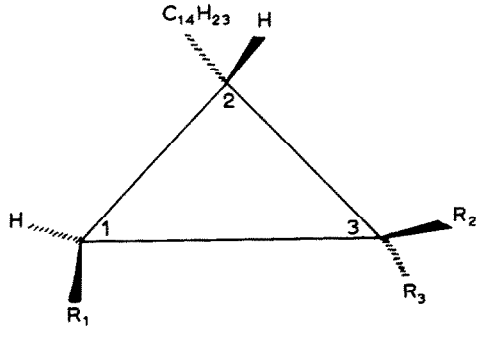
1. Introduction

Presqualene pyrophosphate I, originally isolated from yeast by Rilling [1] has been established as an obligatory intermediate in squalene biosynthesis by both yeast and rat liver preparations [2–4]. The stereochemistry about the cyclopropane ring of presqualene pyrophosphate has been reported as 1*S*, 2*S*, 3*S* as shown in I [5], but more recently there is evidence that the stereochemistry may in fact be 1*R*, 2*R*, 3*R* [6]. Presqualene alcohol III has been synthesised by several workers and in all cases has been converted as the pyrophosphate, into squalene in good yield by yeast preparations [7–9].

Since there has been no report of a similar role for presqualene pyrophosphate in photosynthetic tissue, we now report that a cell-free preparation of *P. sativun* efficiently converts tritiated presqualene pyrophosphate into squalene. Germinating pea seeds (*P. sativum* were chosen, since this system has been used extensive for studying the conversion of mevalonic acid into squalene [10, 11].



Structure	R_1	R_2	R_3
I	CH_2OPP	CH_3	$C_{11}H_{19}$
II	CH_2OPP	$C_{11}H_{19}$	CH_3
III	CH_2OH	CH_3	$C_{11}H_{19}$
IV	CH_2OH	$CH_{11}H_{19}$	CH_3

2. Materials and methods*

2.1. *Preparation of [^{3}H]presqualene pyrophosphate*

Unlabelled and tritiated presqualene alcohols were synthesised as described previously [7]. The labelled material, in which the hydrogens attached to the carbon atom bearing the alcohol function were replaced by tritium, had a specific radioactivity of 89 mCi/μmole.

The tritiated alcohol was phosphorylated using the method of Cornforth and Popjak [12] as modified by Edmond et al. [2]. The reaction residue was extracted with benzene and an aliquot applied to a thin-layer plate (Silica Gel H) developed with propan-2-ol: conc. ammonia (sp. gr. 0.88): water (6:3:1, by vol). The plate was scanned with a 'Panax' radioscanner which revealed that the various phosphorylation products of presqualene alcohol were well separated. The band considered to be the pyrophosphate (R_f 0.35) accounted for 30% of the radioactivity recovered from the reaction flask. To confirm the identity of this band with presqualene pyrophosphate

* Throughout the phosphorylation and incubation procedures silanised glassware was used to reduce the risk of the pyrophosphate adhering to the vessel walls.

the phosphorylation was repeated using [^{3}H]presqualene alcohol diluted with carrier material to a known specific radioactivity, and a known weight of di-triethylammonium phosphate which had previously been equilibrated in acetonitrile with [^{32}P]orthophosphoric acid (NEN Chemicals). After extraction and TLC until radiochemically pure, the band in question had a molecular ratio $^3H/^{33}P$ of 1/1.91, confirming its identity with presqualene pyrophosphate. It was subsequently shown that this band cochromatographed in the above TLC system with a sample of authentic presqualene pyrophosphate kindly donated by Professor H.C. Rilling.

The remaining tritiated presqualene pyrophosphate was isolated from the reaction mixture and chromatographed until essentially radiochemically pure. The tritiated material (overall yield 5%) was stored frozen in benzene and its purity checked immediately prior to use.

2.2. *Incubation of [^{3}H]presqualene pyrophosphate with a cell-free system of P. sativum and isolation of squalene*

The cell-free preparation from peas was based on that of Green and Baisted [11]. Seeds of *Pisum sativum* (variety 'onward') were soaked overnight and then germinated in light, damp conditions. To overcome the risk of missing one of the two optimum germination periods for squalene synthesis [11], seeds of germination times varying between 1 and 5 days were used. The seeds (50) were crushed in a mortar and pestle and homogenised with 0.1 M potassium phosphate buffer pH 7.4 (50 ml) containing 0.4 M sucrose, 5 M glutathione and 5 mM $MgCl_2$. The suspension was filtered through cheesecloth and then homogenised in a "Thomas" homogeniser. The preparation was centrifuged at 40,000 *g* for 10 min and the supernatant used for incubation. All manipulations were carried out at 0°. The protein content of the cell-free preparation was estimated by a modified biuret reaction [13] as 45 mg/ml.

The substrate (1.6×10^5 cpm/tube) was added to the incubation tubes emulsified with 1 mg of Tween 80 in the above buffer, Cofactors were added so that their final conc. were: NADPH, 0.25 mM; NADP, 0.25 mM; glucose-6-phosphate, 2 mM and glucose-6-phosphate dehydrogenase, 1.5 units/tube. Incubations (final vol 11 ml) were started by the addition of the cell-free preparation and were carried out anaerobically at 25° for 1.5 hr. The reaction was stopped by the addition of 10 ml 10% methanolic potassium hydroxide. Following the inclusion of 50 μg of carrier squalene/tube the reaction mixtures were saponified overnight at 5° and then extensively extracted with ethyl acetate. The combined extracts were washed neutral with water, dried and applied to thin layers of Silica Gel G impregnated with rhodamine 6G and developed with petroleum ether (b. range 40–60°). The squalene band (R_f 0.6) was eluted with ether and an aliquot radioassayed.

The remaining squalene was rechromatographed in the same system and the corresponding eluted band divided into two aliquots. One aliquot ($\frac{1}{4}$) was subjected to gas–liquid radiochromatography on a 3% SE-30 column maintained at 220° with an argon carrier gas flow of 60 ml/min. The eluate from the column was split so that 90% could be collected in a capillary tube maintained at ambient temperature whilst the remaining 10% was detected. Capillary tubes were changed at 2 min intervals, eluted with ether, and their contents radioassayed. The second aliquot ($\frac{3}{4}$) was diluted with 45 mg of authentic squalene and purified by the thiourea adduct [14]. The squalene, which was regenerated from the crystalline adduct by treatment with water, was recovered by petrol extraction and its specific radioactivity determined.

3. Results

The radioactivity associated with the squalene band isolated from duplicate incubations of tritiated presqualene pyrophosphate with the *P. Sativum* cell-free system is recorded in table 1. A mean 10% conversion of the substrate into the squalene TLC band was observed. An incubation containing a boiled enzyme blank converted the presqualene pyrophosphate in only 0.5% of the theoretical yield.

In order to confirm the identity of the incubation product, the two squalene TLC bands were pooled and the combined radioactivity rechromatographed in the same system. The band cochromatographing with squalene was eluted and contained greater than 75% of the applied radioactivity. An aliquot of the squalen

Table 1
The incorporation of presqualene pyrophosphate into squalene by *P. sativum* cell free preparation.

Sample	State of enzyme	Conversion (% of theoretical yield)
Test 1	Active	9.0
Test 2	Active	11.1
Control	Boiled	0.5

band was examined by gas-liquid radiochromatography, and the histogram of the results (fig. 1) showed that of the 84% of radioactivity recovered from the column, greater than 95% was associated with the squalene mass peak. The remaining squalene band was diluted with authentic squalene and purified by successive thiourea adduct formation. The specific radioactivity of the regenerated squalene was determined after each purification step and the results are

Table 2
Specific radioactivity of squalene recovered from thiourea adduct formation.

Squalene	Specific radioactivity (cpm/mg)
Initial	259
ex Adduct 1	246
ex Adduct 2	250
ex Adduct 3	241

recorded in table 2. It is apparent that the specific radioactivity of the squalene did not change significantly during purification.

In a separate incubation the isomeric presqualene pyrophosphate II, synthesised from the corresponding alcohol IV, was used as substrate. The conversion into squalene represented only 0.7% of the theoretical yield, the small increase over and above the blank value (0.2%) could be accounted for by the presence of low levels of I in II as revealed by GLC examination

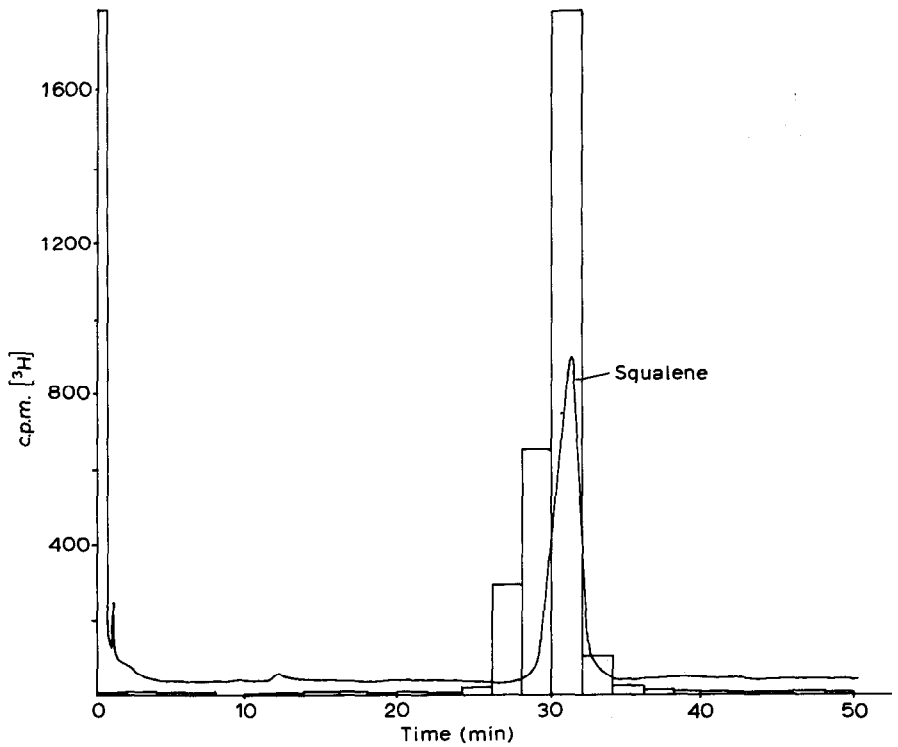


Fig. 1. Gas liquid radiochromatography of isolated squalene band.

of the corresponding tritiated alcohols III and IV prior to phosphorylation.

4. Discussion

The above combined data supports the conclusion that a cell-free system of *P. sativum* will convert tritiated presqualene pyrophosphate I into squalene in 10% yield. The isomeric substrate II is not significantly converted by the same enzymic preparation. It may be concluded, therefore, that the photosynthetic *P. sativum* system contains the biosynthetic machinery to deal with the same isomer of presqualene pyrophosphate that has now been established as an intermediate in squalene biosynthesis by yeast and rat liver preparations. It is tempting to suggest a similar obligatory role for presqualene pyrophosphate in plant systems but such a conclusion will have to await its isolation from plant tissue and its formation enzymatically from labelled farnesyl pyrophosphate.

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